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Targeting the vaccinia virus L1 protein to the cell surface enhances production of neutralizing antibodies☆

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ABSTRACT

The current live-orthopoxvirus vaccine is associated with minor to serious adverse affects, and is contraindicated for use in a significant portion of the population. As an alternative vaccine, we have previously shown that a DNA subunit vaccine (4pox) based on four orthopoxvirus immunogens (L1R, B5R, A27L and A33R) can produce protective immunity against lethal orthopoxvirus challenges in mice and nonhuman primates. Because antibodies are critical for protection against secondary orthopoxvirus infections, we are now interested in strategies that will enhance the humoral immune response against vaccine targets. Here, we tested the immunogenicity of an L1R construct to which a tissue plasminogen activator signal sequence was placed in frame with the full-length L1R gene. The tPA-L1R construct produced a more robust neutralizing antibody response in vaccinated mice when the DNA vaccine was administered by gene-gun as a prime/single boost. When the tPA-L1R construct was substituted for the unmodified L1R gene in the 4pox vaccine, given as a prime and single boost, animals were better protected from lethal challenge with vaccinia virus (VACV). These findings indicate that adding a tPA-leader sequence can enhance the immunogenicity of the L1R gene when given as a DNA vaccine. Furthermore, our results demonstrate that a DNA-based vaccine is capable of establishing protection from lethal orthopoxvirus challenges when administered as a prime and single boost without requiring adjuvant.

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1. Introduction

As a consequence of a worldwide vaccination effort, smallpox as a naturally occurring disease was eradicated in the late 1970s. The threat that variola virus (VARV), the causative agent of smallpox, may be accidentally or maliciously released has led to new interest in vaccinating the military and other "first responders" against orthopoxviruses. This renewed interested in vaccination is further supported by the potential that bioinformatics coupled with synthetic biology could be used to engineer orthopoxvirus-based bioterrorism weapons. This latter threat is substantiated by recent and ongoing studies identifying the subtle genetic differences between orthopoxviruses, in particular VARV, which impact pathogenesis and viral tropism [1–3]. Additionally, naturally occurring monkeypox is an emerging zoonosis [4,5]. Despite being localized to regions of Africa, a monkeypox outbreak recently occurred in the United States [6], representing the potential for worldwide dissemination of this orthopoxvirus.

The current licensed orthopoxvirus vaccine, Dryvax, and the newly licensed ACAM2000 consist of live vaccinia virus (VACV) [7–9]. These vaccines are highly protective and give long-lasting immunity to the vaccinated individual. However, the use of live VACV is associated with a multitude of health risks. These risks range from the potential of spreading the virus to other sites on the body, including the eye, and to non-vaccinated persons in close contact with the vaccinee [7–9]. More serious and life-threatening risks include encephalitis, progressive vaccinia, eczema vaccinatum, myocarditis, and even death [8]. Because of these health risks, vaccination is contraindicated in pregnant women, the immunocompromised, and in persons with dermatological abnormalities, such as eczema [7–9]. Kretzschmar et al. recently reported that the frequency of death associated with vaccination might be higher than previously believed [10]. To diminish these health risks, attenuated VACV viruses, such as modified vaccinia Ankara (MVA) and LC8m16 have been developed [11–14]. However, failed to induce protective immunity in immuno-compromised animals, possibly due to host defects in B-cell antibody class switching [15]. Furthermore, attenuated viruses still encode a multitude of proteins, many of which are involved in immune modulation or have unknown

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14. ABSTRACT
The current live-orthopoxvirus vaccine is associated with minor to serious adverse affects, and is contraindicated for use in a significant portion of the population. As an alternative vaccine, we have previously shown that a DNA subunit vaccine (4pox) based on four orthopoxvirus immunogens (L1R, B5R, A27L and A33R) can produce protective immunity against lethal orthopoxvirus challenges in mice and nonhuman primates. Because antibodies are critical for protection against secondary orthopoxvirus infections, we are now interested in strategies that will enhance the humoral immune response against vaccine targets. Here, we tested the immunogenicity of an L1R construct to which a tissue plasminogen activator signal sequence was placed in frame with the full-length L1R gene. The tPA-L1R construct produced a more robust neutralizing antibody response in vaccinated mice when the DNA vaccine was administered by gene-gun as a prime/single boost. When the tPA-L1R construct was substituted for the unmodified L1R gene in the 4pox vaccine, given as a prime and single boost, animals were better protected from lethal challenge with vaccinia virus (VACV). These findings indicate that adding a tPA-leader sequence can enhance the immunogenicity of the L1R gene when given as a DNA vaccine. Furthermore, our results demonstrate that a DNA-based vaccine is capable of establishing protection from lethal orthopoxvirus challenges when administered as a prime and single boost without requiring adjuvant.

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functions. The potential risk of these factors remains largely unexamined.

As an alternative to live virus vaccines, DNA and/or protein-based-subunit vaccines targeting one or more orthopoxvirus immunogens are being explored. Early studies demonstrated that protein or DNA-expressing A33 or B5 could protect mice from VACV challenge [16]. Recently, it was reported that vaccination with the A33 protein protects mice from challenge with ectromelia virus [17]. Currently, targets of orthopoxvirus subunits vaccines include D8, H3, A33, A27, L1 and B5 [16–24]. We developed a combination DNA vaccine (termed 4pox) that targets four orthopoxvirus antigens (L1, A27, B5 and A33) [21–23]. Orthopoxviruses have two antigenically distinct infectious forms, extracellular enveloped virions (EEV) and intracellular mature virions (IMV) [25]. EEV particles are primary involved in viral dissemination within an infected host, while the more environmentally stable IMV are thought to be involved in spread between hosts. Accordingly, our 4pox vaccine targets multiple proteins on both infectious forms of orthopoxviruses, the IMV (L1 and A27) and the EEV (B5 and A33). Plasmids expressing these genes elicit antibody responses against each protein when delivered to the skin by gene-gun or electroporation [20–23]. Importantly, the 4pox vaccine can protect mice and non-human primates from lethal challenge with VACV or monkeypox virus, respectively [21–23]. Fogg et al. demonstrated that a protein vaccine consisting of these targets can also protect animals from lethal orthopoxvirus challenges [24]. Thus, these combinations of orthopoxvirus targets are effective and valuable targets for a subunit orthopoxvirus vaccine.

The L1 protein is encoded by the L1R gene and is a target of the 4pox vaccine [21–23]. L1 is a myristylated 23–29 kDa membrane protein located on the surface of IMVs and beneath the envelope on EEVs [26,27]. This molecule is highly conserved among the orthopoxviruses. Importantly, the L1 protein is a target of potently neutralizing antibodies [20–23,28–31], making it an attractive target for vaccines and therapeutics. The function(s) of L1 remain unknown. In the absence of L1, particle morphogenesis and formation of infectious virus is blocked, suggesting a role for L1 in IMV assembly [27]. Antibodies against L1 can neutralize viral infectivity, suggesting that L1 may also play a role in particle entry either directly or indirectly [30]. The structure of L1 has been solved and reveals a molecule comprised of a bundle of α-helices packed against a pair of two-stranded β-sheets, held together by four loops [32]. The structure also contains three disulfide bonds that are formed in the cytoplasm by a virus-encoded disulfide bond formation pathway [33]. These disulfide bonds are critical for the interaction of potently neutralizing antibodies [30]. Indeed, the crystal structure of L1 bound by a potently neutralizing antibody MAb-7D11 was recently reported [34]. This structure revealed that potentially neutralizing antibodies bind to a discontinuous epitope consisting of two loop regions held together by a disulfide bond.

We are interested in enhancing the 4pox DNA vaccine such that it will require one or two vaccinations to elicit protection in the vaccinated host. Because antibodies are critical for protection against secondary orthopoxviruses challenges [35–37], we are most concerned with strategies that will enhance the humoral immune responses against the 4pox vaccine targets. Previously, we reported the generation of a modified full-length L1R construct where a tissue plasminogen leader sequence (tPA) was placed on the 5′-end of the L1R gene [23]. This modified construct leads to the surface expression of the L1 protein, allowing for an in vitro flow cytometry-based assay to detect anti-L1 antibodies in vaccinated animals. We observed a marked increase in amount of L1 able to interact with conformation-dependent monoclonal antibodies, indicating proteins expressed from the tPA-L1R gene are folded more natively [23]. In this report, the immunogenicity of unmodified and modified L1R DNA vaccines, pWRG/L1R and pWRG/tPA-L1R were tested in mice vaccinated by gene-gun. We found that tPA-L1R produced a higher amount of neutralizing antibodies and provided superior protection in vaccinated mice than unmodified L1R when it was combined with the other 4pox immunogens. We also found that the 4pox DNA vaccine completely protected mice from lethal challenge with VACV when given as a prime and single boost and this protection was improved by substituting unmodified L1R with the tPA-L1R immunogen.

2. Materials and methods

2.1. Cells and viruses

VACV Connaught vaccine strain (derived from the New York City Board of Health strain), VACV strain WR (ATCC VR-1354), and VACV strain IHD-J (obtained from Dr. Alan Schmaljohn) were all maintained in VERO cell (ATCC CRL-1587) monolayers grown in Eagle's minimal essential medium, containing 5% heat-inactivated fetal bovine serum (FBS), 1% antibiotics (100 U/ml penicillin, 100 μg/ml of streptomycin, and 50 μg/ml of gentamicin), 10 mM HEPEs (cEMEM). COS-7 (COS) cells (ATCC CRL-1651) were used for transient expression experiments. BSC-1 cells (ATCC CCL-26) were used for plaque reduction neutralization assays (PRNT). Both BSC-1 and COS cells were also maintained in cEMEM.

2.2. Cloning

The generation of pWRG/tPA-L1R was previously described [23]. Essentially the L1R open reading frame was subcloned into the NheI and BglII sites of pWRG/tPA vector. This vector contains the tPA leader sequence. To construct the pWRG/tPA-A27L, the A27L open reading frame was subcloned into the NheI and BglII sites of pWRG/tPA. A27L was amplified by PCR using the forward primer 5′-GGGGGGCTAGCATGGACGGAACTCTTTTCCCCGG-3′ and the reverse primer 5′-GGGAGATCTTTACTCATATGGACGCCGTCC-3′. These primers contain a NheI and BglII site, respectively. The resultant PCR product was cut with NheI and BglII, gel purified, and ligated into pWRG/tPA, in frame with the tPA signal sequence. Sequence analysis confirmed that the A27L insert was in frame with the tPA signal sequence.

2.3. DNA vaccination with gene-gun

The DNA vaccination procedure has been described [20,38]. Briefly, plasmid DNA was precipitated onto ~2-μM diameter gold beads at a concentration of 1 μg DNA/1 mg of gold. This DNA–gold mixture was coated on the inner surface of irradiated Tefzel tubing and the tubing was cut into 0.5-in. cartridges. Each cartridge contained ~0.25–0.5 μg of DNA coated on 0.5 mg of gold. All cartridges were quality controlled to ensure the presence of DNA. For vaccinations, the abdominal fur of BALB/c mice was shaved and DNA-coated gold was administered using a gene-gun (Powderjet delivery device, Powderject, INC) and compressed helium at 400 p.s.i. to non-overlapping sites. Mice vaccinated with single genes were vaccinated with two cartridges containing the respective gene (0.5–1.0 μg of DNA/dose) at both the prime and boost. Mice receiving multiple genes were vaccinated with one cartridge for each gene at non-overlapping sites (0.25–0.5 μg of DNA/gene/dose) at both the prime and boost. In all experiments, the boost was preformed 3 weeks after the priming vaccination. All mice were at least 7–9-weeks-old at the start of vaccination.

2.4. Scarification

Scarification was preformed by placing a 10-μl drop of phosphate-buffered saline (PBS) containing 8×10^6 PFU of VACV (Connaught) near the base of the tail on each mouse. The tail was then scratched ~15–20 times using a needle on a tuberculin syringe.

2.5. ELISA using purified proteins

Histidine-tagged VACV antigens L1 (300 ng/well), A33 (50 ng/well), B5 (50 ng/well), and A27 (50 ng/well), produced in *Escherichia coli* or mammalian cells (B5 antigen produced in baby hamster kidney cells) and purified on nickel columns, were diluted in 0.1 M carbonate buffer [pH 9.6] and plated in duplicate in the wells of a high-binding, 96-well plate (Corning; Corning, NY). The constructs used to produce these ELISA antigens will be reported elsewhere. To control for background, plates were also coated with an irrelevant protein purified over nickel columns (botulinum toxin at 50 ng/well). Plates were blocked for 1 h with PBS with 0.05% tween (PBS-T) and 5% milk. Mouse sera were serially diluted 10-fold (starting from 1:100) in PBS-T containing 5% milk and *E. coli* lysate. Serum dilutions were incubated with the purified VACV antigens for 1 h at 37 °C. Plates were washed four times in PBS-T and incubated with an anti-mouse IgG conjugated to horseradish peroxidase (HRP) (Sigma) (1:1000) for 1 h at 37 °C. Plates were washed again four times in PBS-T and 100 μl of 2,2′-azinobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) substrate (KPL, Gaithersburg, MD) was added to each well. Reactions were stopped by adding 100 μl of ABTS stop solution of 5% (w/v) sodium dodecyl sulfate. The optical density (O.D.) at 405 nm was read on a Spectramax ELISA plate reader (Molecular Devices; Sunnyvale, CA). End-point-titers were determined as the highest dilution with an absorbance value greater than the mean absorbance value from negative control plasmid-vaccinated animals plus three standard deviations. Mean titers for individual mice were plotted ± standard deviation.

2.6. IgG isotype ELISA

Antibody isotyping was determined for mice vaccinated with L1R, tPA-L1R, A27L, and tPA-A27L with purified L1 or A27 protein essentially as described above. To determine the subclass, secondary antibodies (conjugated to HRP) against IgG1 (1:1000) and IgG2a (1:1000) (Bethyl laboratories; Montgomery, TX) were incubated with duplicate plates. The ratio of IgG1/IgG2a was calculated and graphed.

2.7. PRNT

The plaque-reduction and neutralization assay (PRNT) was described previously [20]. Briefly, VACV strain IHD-J was diluted in cEMEM to give ~250 plaque-forming units (pfu)/ml. Aliquots of this viral suspension (100 μl) were incubated with an equal volume of serum diluted in cEMEM for 1 h at 37 °C, then 180 μl of sample was adsorbed to confluent BSC-1 cell monolayers in 6-well plates for 1 h in a 37 °C 5% CO_2 incubator. All serum samples were heat activated at 56 °C for 30 m before being diluted. Plates were rocked ~15 m. After adsorption, a 2-ml semisolid overlay (Earle's basal minimal essential medium, 1.5% methyl cellulose, 5% heat inactivated FBS, antibiotics (100 U/ml penicillin, 100 μg/ml of streptomycin, and 50 μg/ml of gentamicin) was added to each well. After 4 days in a 37 °C 5% CO_2 incubator, cell monolayers were stained with 1 ml of a staining solution (3% crystal violet and 15% ethanol in H_2O). Plaques were counted and the percent neutralization was calculated relative to the number of plaques in the absence of antibody. Titers represent the reciprocal of the highest dilution resulting in a 50% reduction in the number of plaques. Mean neutralization titers for individual mice were plotted ± standard deviation.

2.8. Viral challenges

Five weeks after the boost, mice were anesthetized and weighed before intranasal administration with a plastic pipette tip containing 50 μl of PBS with 2×10^6 pfu of VACV strain IHD-J. This dose is three times the LD_{50}. Subsequently, mice were observed and weighed daily for 14 d. Moribund mice (>30% body weight) were euthanized.

2.9. Statistical analysis

The statistical significance of ELISAs and PRNTs were determined using the unpaired two-tailed Student's *t* test. Percent weight loss data comparisons between 4pox and 4pox (tPA-L1R) vaccinated mice were also examined by the unpaired two-tailed Student's *t* test at each day postinfection. Significance levels were set at a *p* value less than 0.05.

3. Results

3.1. A tPA leader sequence enhances the neutralizing antibody responses against L1

We previously showed that adding a tPA leader sequence to the L1R gene leads to an enhanced interaction of the L1 molecule with conformationally dependent and potently neutralizing antibodies (MAb-10F5 and MAb-7D11) [23]. Based on these findings, we conducted an experiment to determine if this construct could generate a more robust neutralizing antibody response in mice vaccinated by gene-gun. Groups of eight BALB/c mice were vaccinated by gene-gun with DNA encoding either unmodified L1 or tPA-L1. Three weeks after the prime, mice were boosted. Anti-L1 antibody responses were assessed by ELISA with purified L1. Antibody responses in mice vaccinated with the unmodified L1R gene were below the level of detection after the priming vaccination (Fig. 1A). In contrast, mice vaccinated with tPA-L1R DNA had detectable anti-L1 antibodies after the prime, with a titer of ~2. After the boost, antibody responses induced by both modified and unmodified L1 increased to 3.7 and 2.6 log titers, respectively. The anti-L1 response in tPA-L1R vaccinated animals was significantly greater ($p < 0.05$) than mice vaccinated with unmodified L1R after both the prime and the boost.

We also tested the capacity for anti-L1 antibodies to neutralize VACV in a PRNT. The 50% neutralizing titers (PRNT50s) correlated with the titers observed in the ELISA. After the initial vaccination, PRNT50s for both groups were below the level of detection (Fig. 1B). There was significant neutralizing activity associated with the serum from tPA-L1R-vaccinated mice after the boost and the mean PRNT50 titer for this group was over 550. The mean PRNT50 titer in the unmodified L1R-vaccinated mice was ~14-fold lower. This difference was significant ($p < 0.05$). These findings clearly indicated that adding the tPA leader sequence greatly enhanced the neutralizing antibody response against L1.

3.2. The tPA leader sequence does not enhance production of neutralizing antibodies against the A27 protein

Adding a tPA leader sequence enhances the immunogenicity of several DNA vaccine immunogens, including the orthopoxvirus molecule D8L [19]. Therefore, we tested the capacity of the tPA leader sequence to enhance the antibody response against A27, a protein on the surface of IMVs, beneath the envelop of EEVs

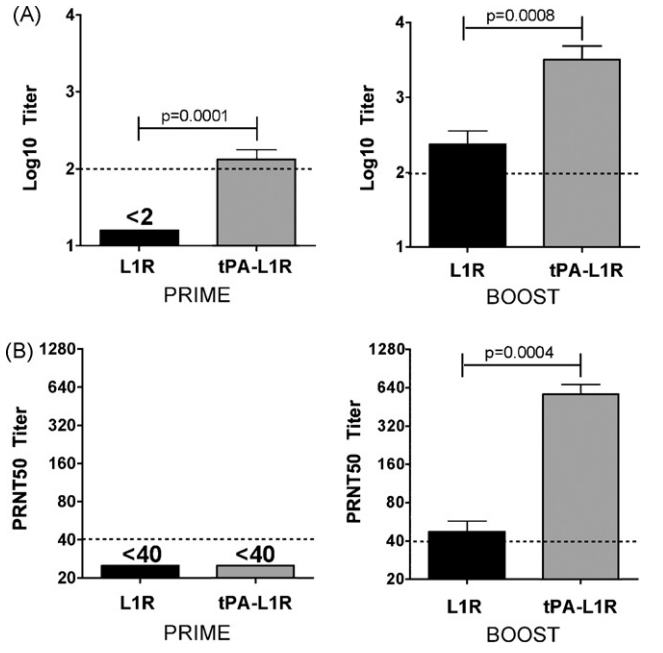

Fig. 1. Antibody responses against L1 in L1R- and tPA-L1R-vaccinated mice. (A) Purified L1 was plated in the wells of a 96-well plate (300 ng/well) in carbonate buffer. Serum from mice vaccinated (prime or boost as indicated) with either L1R or tPA-L1R DNA was serially diluted 10-fold (from 1:100) and incubated with purified protein. Plates were then incubated with a secondary anti-mouse antibody (1:1000) conjugated to HRP. ABTS was added to each well and reactions were terminated using 5% SDS. Endpoint titers were calculated as described in Section 2. Data were plotted as a mean titer for each group ± standard deviation. (B) Serum from L1R or tPA-L1R vaccinated animals (prime or boost as indicated) was serially diluted 2-fold and incubated with ~50 pfu of VACV strain IHD-J for 1 h at 37 °C. Antibody-virus mixtures were adsorbed to confluent monolayers of BSC-1 cells for 1 h at 37 °C. After adsorption, a 1:1 mixture or 2× EBME and 3% methyl cellulose was added to each well. Three days postinfection, plaques were visualized by staining monolayers with 1.5% crystal violet. 50% neutralization titers were calculated relative to the plaque count for virus that was not incubated with serum. Data were plotted as a mean titer for each group ± standard deviation. The lowest dilution tested was 1:40 (dashed-line indicates limit of detection).

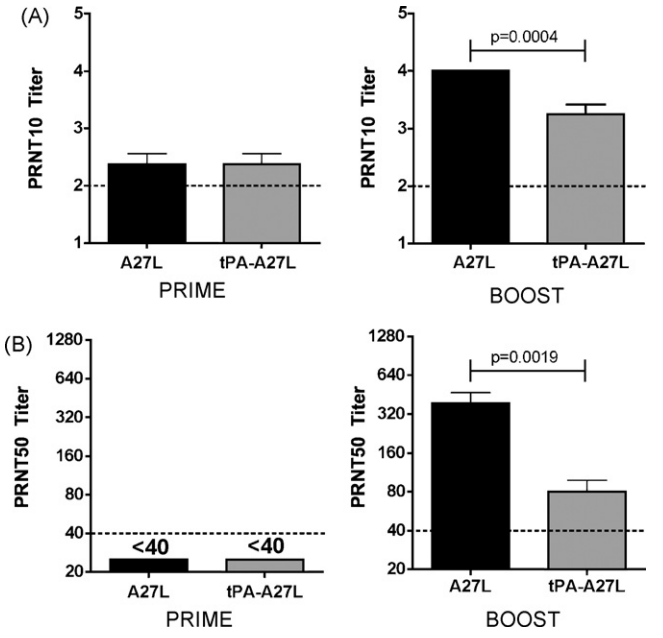

Fig. 2. Antibody responses against A27L in A27L and tPA-A27L in vaccinated mice. (A) Purified A27 (50 ng/well) was plated in 96-well plates. ELISAs were preformed using serum from mice vaccinated with A27L or tPA-A27L DNA as described in the legend to Fig. 1A. (B) The $PRNT_{50}$ was done as described in Fig. 1B, except the serum used was from A27L- and tPA-A27L DNA-vaccinated mice.

[39] and a component of our 4pox vaccine [21]. As determined by ELISA with purified A27, after the initial vaccination there was little difference in anti-A27 antibody responses between mice vaccinated with either A27L or tPA-A27L, and both groups had titers ~2.2 (Fig. 2A). This level of anti-A27 antibody did not neutralize virus (Fig. 2B). Similar to the L1R and tPA-L1R groups (Fig. 1A), ELISA titers for both A27L- and tPA-A27L-vaccinated mice increased after the boost. However, A27L-vaccinated mice had antibody titers that were ~0.5 logs higher than those of the tPA-A27L-vaccinated mice. This difference was statistically significant ($p < 0.05$). This more robust anti-A27 antibody response correlated with a more significant $PRNT_{50}$ titer ($p < 0.05$), which was ~320 compared to the $PRNT_{50}$ titer of tPA-A27L-vaccinated mice, which was below 80. These findings demonstrated that enhanced antibody responses gained by adding the tPA leader sequence to the L1R gene, did not occur with A27L.

3.3. Adding the tPA leader does not impact the IgG isotype against L1

Typically, epidermal vaccination by gene-gun leads to a Th2 response characterized by the production of IgG1 antibodies [40]. As such, trafficking of L1 from the cytoplasm to the secretion pathway, which targets molecules through the endoplasmic reticulum and golgi, could have affected the type of immune responses elicited against this molecule. Therefore, it was possible that in animals vaccinated with tPA-L1R, there might be a more significant activation of the Th1 arm of the immune response. To investigate this possibility, serum from mice vaccinated with tPA-L1R or unmodified L1R DNA was incubated with purified L1 and then incubated with an isotype-specific secondary antibody. As shown in Fig. 3, there was little change in the ratio of IgG1 to IgG2a antibodies between L1R and tPA-L1R vaccinated mice. Antibody responses in both groups were skewed towards a IgG1 response, whereas a more balanced response was observed using hyperimmune serum from mice infected with VACV (Fig. 3). Thus, adding the tPA leader sig-

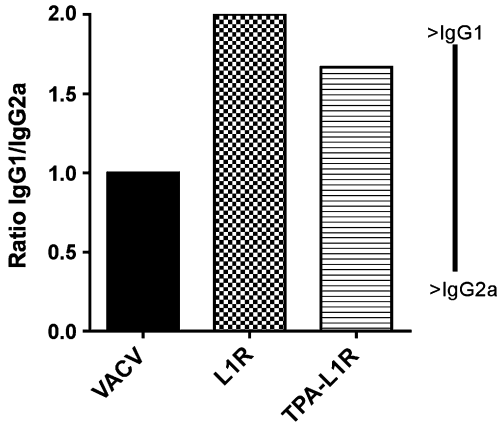

Fig. 3. IgG isotypes in vaccinated animals. Pooled serum from either VACV-infected mice or mice vaccinated with L1R, tPA-L1R, A27L, and tPA-A27 was serially diluted 10-fold. Dilutions were incubated with two plates each containing either purified L1 (L1R and tPA-L1R vaccinated mice) or purified A27 (A27L- and tPA-A27L-vaccinated mice). Secondary anti-mouse antibodies conjugated to HRP and specific for either IgG1 or IgG2a were then incubated with the samples. ABTS was added to each well and reactions were terminated using 5% SDS. Endpoint titers for each secondary antibody were calculated as described in Section 2. The ratio of IgG1 to IgG2a was determined and plotted.

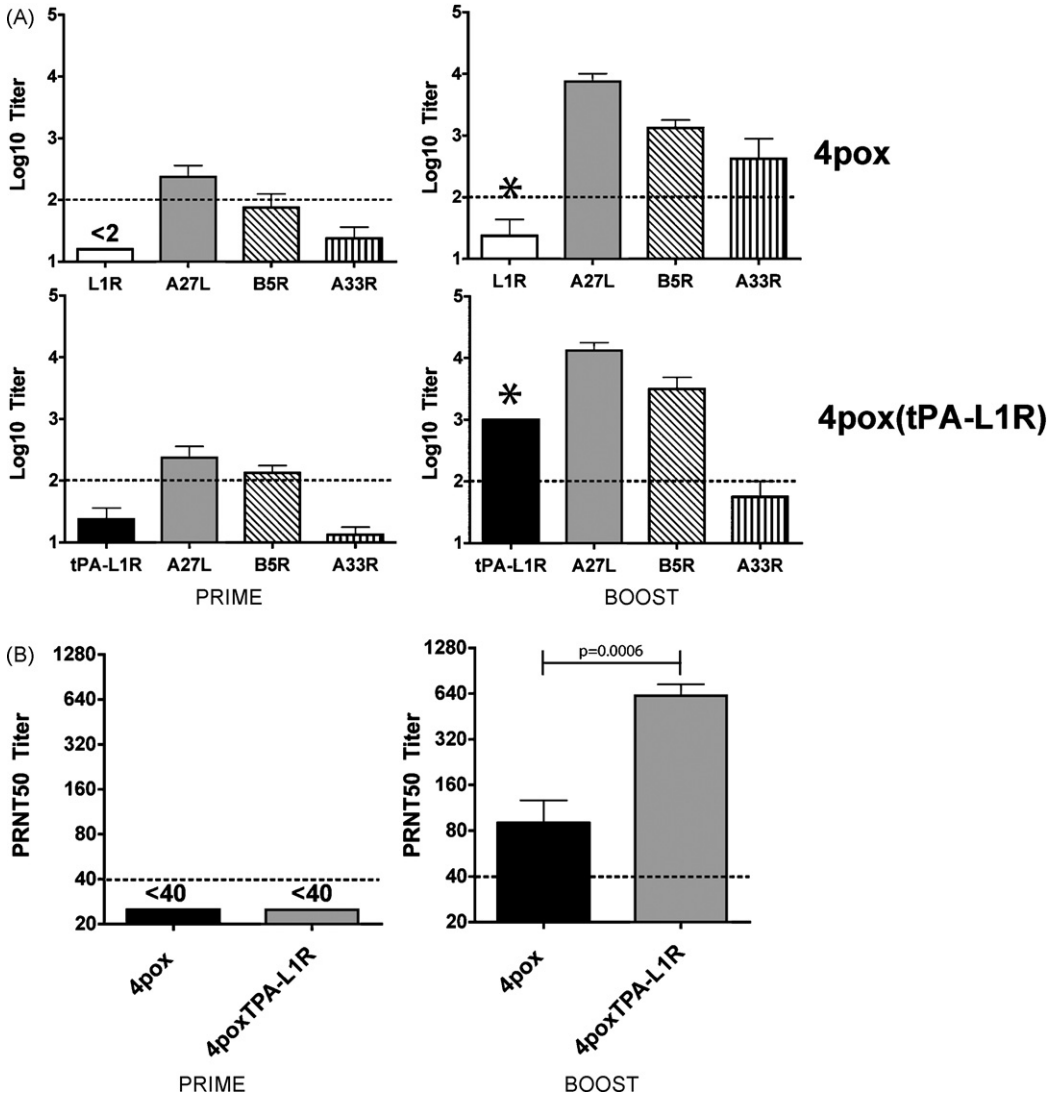

Fig. 4. Antibody responses against B5, A33, A27 and L1 in DNA vaccinated mice. (A) Mice were primed with all four immunogens (B5R, A33R, A27L, and L1R or tPA-L1R) and boosted 3-weeks later with the same molecules as indicated. Serum from vaccinated animals (prime and boost) was examined by ELISA for reactivity with all four immunogens. Serum from mice was serially diluted 10-fold and incubated with immunogens as described in Fig. 1A. The asterisk denotes that the anti-L1 antibody response was significantly greater ($p = 0.00002$) for the 4pox (tPA-L1R) group following the boost; all other antibody responses were statistically insignificant ($p > 0.05000$). (B) The presence of neutralizing antibodies in mice vaccinated with all four antigens was assessed by PRNT. The lowest dilution tested was 1:40 (dashed-line indicates limit of detection).

nal to L1R does not impact isotype preference generated following gene-gun delivery.

3.4. The tPA leader sequence enhances antibodies against L1 in mice vaccinated with a multi-gene combination

To ensure adequate cross-protection and reduce the potential for a genetically modified virus to bypass protection of a subunit vaccine, our candidate molecular vaccine targets multiple antigens present on both infectious forms of orthopoxviruses, the EEV (A33 and B5) and IMV (L1 and A27). Therefore, we next examined if there was an enhancement in neutralizing antibody production against L1 when the unmodified L1R gene was substituted with tPA-L1R in mice vaccinated by gene-gun against all four antigens in mice (4pox vaccine). Mice were primed with DNA encoding all four immunogens (A33, B5, A27 and L1, or tPA-L1) and then boosted 3 weeks later using the same combinations. Antibody responses against the four targets were then assessed by ELISA with purified VACV proteins. We observed that L1 antibody responses in mice vaccinated with 4pox were below the level of detection after the prime and only two mice developed detectable antibodies against L1 after the boost (Fig. 4A and Table 1). Antibody responses against the other three antigens were detected after the boost (Fig. 4A and Table 1). Among these responses, A27 was the most robust, while A33 was the weakest. After the initial vaccination, three mice in the 4pox (tPA-L1R) group had detectable L1 antibody responses and all mice developed anti-L1 titers of 3 logs after the boost (Fig. 4A and Table 1). Antibody titers against B5 and A27 were also detectable in mice after the boost. However, some mice did not develop anti-A33 responses and even after the boost, three of the eight mice had anti-A33 responses below the level of detection (Table 1). Of these data, only the anti-L1 response after the boost was significantly different between the 4pox and 4pox (tPA-L1R) groups ($p < 0.05$). Responses against B5, A27 and A33 were all found to be statistically insignificant ($p > 0.05$).

The neutralization responses elicited by 4pox or 4pox (tPA-L1R)-vaccinated mice were also evaluated. Neither the 4pox nor the 4pox

Table 1
Immunogenicity data for individual mice vaccinated with 4pox or 4pox (tPA-L1R)[a]

Mouse ID	Prime, ELISA[b]					Boost, ELISA[b]				
	PRNT50	L1	A33	B5	A27	PRNT50	L1	A33	B5	A27
4pox										
773	20	1	1	2	2	80	1	3	3	4
815	20	1	1	2	2	20	1	2	3	3
775	20	1	2	2	2	40	1	3	3	4
776	20	1	2	2	3	320	3	4	4	4
777	20	1	1	3	2	20	2	3	3	4
778	20	1	1	2	2	40	1	1	3	4
779	20	1	2	1	3	160	1	3	3	4
780	20	1	1	1	3	40	1	2	3	4
4pox (tPA-L1R)										
781	20	2	1	2	3	640	3	2	4	5
782	20	1	1	3	2	1280	3	2	4	4
783	20	2	1	2	2	640	3	1	3	4
784	20	2	1	2	2	640	3	2	3	4
785	20	1	1	2	3	160	3	3	3	4
786	20	1	1	2	2	640	3	1	4	4
787	20	1	2	2	3	640	3	2	3	4
788	20	1	1	2	2	320	3	1	4	4

[a] Mice were vaccinated with the indicated genes delivered on separate cartridges. Mice were vaccinated with one cartridge per gene.
[b] ELISAs were performed using the purified protein for each orthopoxvirus antigen.

(tPA-L1R) had a $PRNT_{50}$ titer after the prime. However, neutralizing responses were observed for both groups after the boost (Fig. 4B and Table 1). Among these responses, the $PRNT_{50}$ was ~7-fold higher in mice vaccinated with 4pox (tPA-L1R). This was a significant difference ($p < 0.05$). These findings demonstrated that when tPA-L1R was substituted for the unmodified L1R gene, the 4pox DNA vaccine elicited antibody responses against L1 and the three other antigens (B5, A33 and A27) after only two vaccinations.

3.5. Improved protection in mice vaccinated with a multi-gene combination containing tPA-L1R

Mice were vaccinated two times with L1R, tPA-L1R, 4pox, or 4pox (tPA-L1R) and then challenged intranasally with three LD_{50} of VACV strain IHD-J. For controls, two groups of mice either unvaccinated or vaccinated with live-virus (Connaught) by tail scarification were also challenged. Weights were monitored for 14 days postinfection. As shown in Fig. 5, unvaccinated mice began to lose weight on day 2 and by day 7, all mice died. Mice vaccinated with unmodified L1R also began to lose weight on day 2 and all mice succumbed to infection by day 7. Mice vaccinated with tPA-L1R survived longer; nevertheless, all mice in this group died by day 11. We observed greater protection in mice vaccinated with multi-gene combinations compared to those vaccinated with single genes. Mice vaccinated with the 4pox vaccine had a transient loss in weight reaching a maximum of ~18% by day 5. Weight of the 4pox-vaccinated mice started to increase on day 7; however, on day 14 weight remained ~11% below starting weight. Mice vaccinated with 4pox (tPA-L1R) were better protected from VACV challenge (Fig. 5). Weight loss for this group was less severe compared to that of the 4pox group and by day 14, weights in this group were only 5% below starting weight. The difference in weight loss between the 4pox and 4pox (tPA-L1R) groups were significant from day 3 to day 14 ($p < 0.05$). As expected, mice vaccinated with live virus had a very transient weight loss that was maximal at day 4 and all the mice survived infection. These findings demonstrated that mice vaccinated with the 4pox molecular vaccine were better protected from lethal challenge with VACV when the tPA-L1R gene was substituted for the unmodified L1R gene.

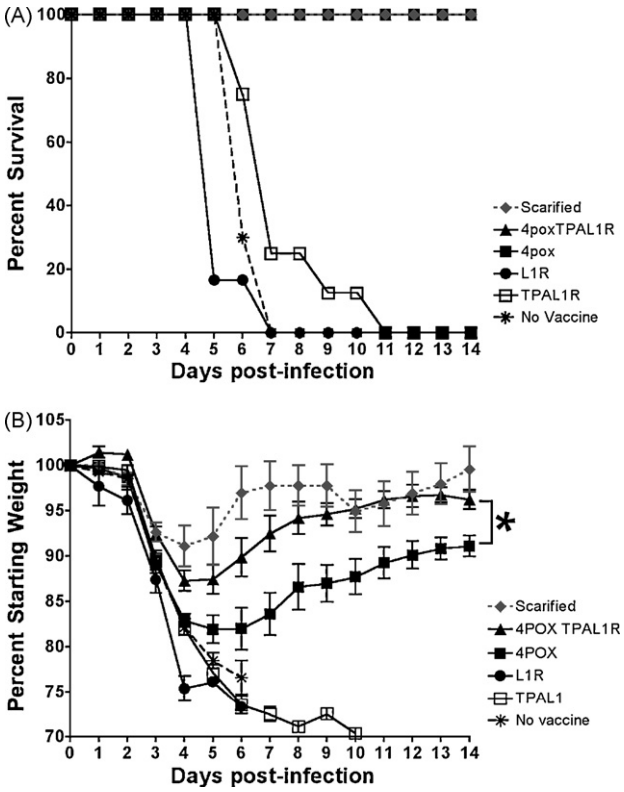

Fig. 5. VACV challenge of vaccinated mice. Groups of mice vaccinated twice by gene-gun with the indicated DNA vaccine or once by tail scarification with live VACV were challenged with 2×10^6 pfu of VACV strain IHD-J. Mice were weighed daily for 14 days. (A) Survival curves for each group are shown. 100% of the mice in the scarified, 4pox (tPA-L1R), and 4pox survived. (B) The percent weights of surviving mice were calculated relative to starting weights (day 0) and plotted. The differences in weight loss between the 4pox and 4pox (tPA-L1R) groups were significant for every time point starting on day 3 ($p < 0.05$) as denoted by the asterisk.

4. Discussion

Antibodies are critical for protection against secondary orthopoxvirus infections [35,36]. Thus, we are interested in enhancing our 4pox subunit vaccine such that it will elicit potent humoral responses after one or two vaccinations. We previously reported that adding a tPA leader sequence significantly increased the interaction of surface-expressed L1 with potently neutralizing monoclonal antibodies [23]. Here, we demonstrated that use of the tPA-L1R construct as a DNA-vaccine led to enhanced neutralizing antibody responses against the L1 protein in mice vaccinated by gene-gun. The neutralizing antibody responses in vaccinated mice were significantly higher than those determined in our previous studies, where mice vaccinated with L1R DNA had geometric mean titers of 235 and 101 after three vaccinations [20,21]. By adding the tPA leader sequence to L1R, we were able to generate neutralizing antibody responses with a GMT titer of 489. Importantly, this high neutralization response was elicited after only two vaccinations.

L1 contains three disulfide bonds normally formed by a virus-encoded disulfide bond formation pathway [32,33]. The requirement for disulfide bonds in the interaction of several potently neutralizing monoclonal antibodies, including MAb-7D11 and MAb-10F5, has been reported [30,34]. However, the precise structural role of these bonds in formation of the antibody epitope(s) was not clear until recently when the structural basis for the binding of potentially neutralizing antibodies to the L1 protein was reported [34]. These data reveal a discontinuous epitope containing two loops bound by a single disulfide bond. These findings sup-

port a conclusion that in the absence of the virus-encoded disulfide bond formation pathway (such as in a transfected cell), the majority of L1 folds improperly and the critical antibody epitope is not formed. Improper folding was likely responsible for the low anti-L1 responses generated in our previous studies [20,21]. It also provides a reason as to why other groups have been unable to generate anti-L1 responses with potent neutralizing activity when vaccinating with L1R DNA [41]. We speculate that the capacity of the tPA leader to traffic molecules through the endoplasmic reticulum (ER) is the mechanism by which the tPA leader enhances the generation of neutralizing monoclonal antibodies in tPA-L1R vaccinated animals. Within the ER, L1 can usurp the host disulfide bond formation machinery and thereby fold correctly. Consequentially, preservation of the critical epitope would permit generation of potently neutralizing antibodies. We observed that enhanced interaction of tPA-L1 with neutralizing antibodies did not seem to be specific to the tPA signal, as adding an IgG kappa leader sequence also led to increased interaction of L1 with neutralizing antibodies in transfected COS cells (data not shown). To generate L1 for the purposes of protein purification, Aldaz-Carroll et al. used a similar approach and targeted L1 lacking its transmembrane (TM) region to the secretory pathway in insect cells using a melittin signal sequence [42]. Secreted L1 (lacking the TM region) appears to fold more natively as evidenced by its ability to interact with conformationally dependent antibodies [42].

The addition of the tPA leader sequence has been employed to enhance antibody responses against numerous antigens, including the orthopoxvirus D8L gene and the Japanese encephalitis virus envelope protein [19,43]. For DNA vaccine immunogens, typically the TM regions of the antigen are removed to allow secretion of the molecules into the extracellular milieu. Contrary to this convention, the TM region of L1 was purposefully retained. This was done for several reasons. We found that a secreted version of L1 containing only the ectodomain region did not interact with potently neutralizing antibodies to the same extent as full-length L1, despite being expressed at similar levels (data not shown). Furthermore, Aldaz-Carroll et al. were unable to generate neutralizing antibodies that interacted with the epitope recognized by potentially neutralizing antibodies such as MAb-7D11 or 2D5 in mice vaccinated with purified L1 lacking the TM [42]. Instead, they identified less potent neutralizing antibodies interacting with different epitopes. The failure to generate antibodies interacting with the discontinuous epitope may possibly have been due to glycosylation of the L1 molecule by insect cells. However, removal of the TM region can negatively impact the immunogenicity of other antigens targeted through the secretory pathway. For example, Rath et al. found that the presence of the TM domain and a secretion signal sequence on the rabies virus glycoprotein was needed to obtain the highest levels of neutralizing antibody [44]. This is because in the absence of the TM, the protein fails to fold properly leading to the disruption of the critical epitope [44]. Hence, for antigens whose antibody epitopes are highly conformationally dependent, such as L1 [34], retention of the TM region might be critical.

It was recently reported that the tPA leader sequence can enhance the humoral responses against the orthopoxvirus D8L gene [19]. The authors found a slight improvement in the protective capacity of tPA-D8L lacking the TM versus D8L. This report, combined with the fact that the tPA leader sequence enhanced the production of antibodies against L1 (Fig. 1) suggests adding this sequence to all of our 4pox vaccine targets would be beneficial. This is further supported by numerous reports demonstrating the positive effects the tPA leader sequence can have on other antigens [43–48]. However, the ability of the tPA leader sequence to enhance neutralizing antibody production appears to be antigen specific. This conclusion is based from our finding that tPA-A27L did not elicit any increase in antibody responses against the A27 protein (Fig. 2). In fact, we saw about a 0.5 log decrease in ELISA titer and a marked decrease in $PRNT_{50}$ titers when the tPA-A27L was used for vaccination (Fig. 2). It is not clear why tPA-A27L did not lead to enhanced antibody production. A27 is normally found in the cytoplasm of infected cells. Perhaps targeting A27 to the ER modifies the antigen, possibly by glycosylation, such that important epitopes are disrupted.

In a previous study, we found that unmodified L1R could protect ~60% of mice against lethal challenge with VACV strain WR after three vaccinations [20]. In this current study, the L1R group had low $PRNT_{50}$ titers and all mice died from infection. In contrast, the tPA-L1R group had $PRNT_{50}$ titers significantly higher than in previous studies (Fig. 1 and [20,21]). However, despite these high titers, animals in the tPA-L1R group, while surviving a few days longer than the L1R group, all succumbed to infection (Fig. 5). In previous studies, mice were challenged by the intraperitoneal route with VACV strain WR [20,21], whereas in this current study mice were challenged i.n. with VACV strain IHD-J. The latter model involves a lower challenge dose and requires more *in vivo* dissemination of virus as evidenced by the extended time course of disease. IMV neutralizing antibodies are insufficient to protect in this model unless the response is potent enough to reduce the initial exposure below a critical level (Hooper, unpublished data). If this does not occur, then IMV-infected cells produce EEV, and this progeny virus disseminates unabated resulting in lethal disease, usually by day 6. If anti-EEV antibodies are present, then it is believed that dissemination is prevented and protection is achieved.

Indeed, the inability of vaccination against L1 alone to protect against lethal infection highlights the importance of targeting both infectious forms of orthopoxviruses. We, and others, have shown that vaccines comprised of combinations of immunogens targeting both infectious forms of orthopoxvirus, IMV and EEV, provides superior protection versus targeting only IMV or EEV [19–24,41,49,50]. IMV neutralizing antibodies, including those against L1 and A27, neutralize virus in the initial exposure and also can eliminate any IMV released from infected cells that are lysed or EEV that are disrupted. It is unclear how anti-EEV antibodies protect, including those against A33 and B5, however the mechanism(s) likely involves the prevention of EEV spread within the host and possibly the elimination of infected cells by processes involving complement and/or Fc-receptor bearing cells. In previous studies, protection elicited by a protein or DNA vaccine targeting both infectious forms required three or four vaccinations. Recently, it was shown in mice that purified L1 and A33 proteins could elicit a protective antibody response after only two vaccinations if certain adjuvants were used [49]. Here, we demonstrated that delivery of the 4pox antigens, in the absence of adjuvants, could generate protective immune responses after only two vaccinations (Figs. 4 and 5 and Table 1). This is the first report demonstrating that a subunit orthopoxvirus vaccine can protect against lethal challenge after only two vaccinations, without requiring the addition of adjuvant. Moreover, substitution of tPA-L1R for unmodified L1R DNA provided statistically greater protection from challenge (Fig. 5). The enhanced protection was probably attributable to higher amounts of anti-L1 capable of neutralizing input virus and lowering the initial viral load in infected animals.

5. Summary

The L1 molecule is an ideal target for pan-orthopoxvirus subunit vaccines and therapeutics because it is a target of potently neutralizing antibodies [20,21,23,28–30,42], it is absolutely essential for replication [27], and the protective epitope is conserved between orthopoxviruses. Because L1 produced in transfected cells

does not have access to an essential virus-encoded disulfide bond formation pathway, much of the protein likely folds incorrectly. By targeting the recombinant L1 to the ER, it was possible to generate L1 that was folded more natively as indicated by interaction with conformationally dependent, neutralizing monoclonal antibodies. This tPA-L1R construct was capable of enhancing neutralizing antibody responses in mice vaccinated by gene-gun. When the modified L1R construct was used in the 4pox vaccine, we observed increased protection of vaccinated animals after a prime and single boost.

In addition to using tPA-L1R as a component in our DNA vaccine, we are also examining if this construct can be used to generate neutralizing antibodies in systems aimed at producing molecules suitable for use in humans. These molecules could then be used as therapeutics or postexposure prophylactics to treat orthopoxvirus infections and/or adverse effects related to vaccination with live-virus.

Disclosure

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles state in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

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